Exhibit C

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ANTIBODIES AGAINST SYNTHETIC PEPTIDES

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INTRODUCTION

The introduction of a simple and efficient peptide synthesis methodology in the early 1960's (1) put a powerful research tool at the disposal of biologists and protein chemists alike. Thus, it was immediately feasible to synthesize not only biologically active peptides, but also discrete segments of proteins. It was obvious from the start, also, that this technology could be coupled with longstanding immunologic procedures whereby small molecules can be used as antigenic materials (2). Indeed, the approach was soon applied to a number of proteins in a quest to find what features made them antigenic (3). It is fair to say, though, that the full capacity of the combined immunologic and synthetic techniques was never exploited during the 1960's and 1970's. Since then advances in the field of DNA sequencing have provided a plethora of sequence information about proteins, real and putative, many of which have never been isolated, never mind characterized. The sequence data can be utilized in a variety of ways, especially in conjunction with computer-aided predictive schemes that allow a degree of meaningful conjecture about the three-dimensional structure of a given sequence. Among the ends to which DNA/protein sequence data can be put is the chemical

synthesis of peptides corresponding to portions of the protein. Antibodies can be raised against these peptides, and these in turn can be used as tools for identifying and isolating the proteins, as well as localizing them in vivo or characterizing them in vitro. These synthetic peptides can also be used as traditional immunizing agents for protection against viruses, bacteria, plasmodia or toxins. In this article we review briefly the history of synthetic peptides in this area of immunology and the manifold uses to which antibodies to them have been and can be put.

The first use of an isolated determinant for raising antibodies that recognized the corresponding intact protein was reported by Anderer in 1963 (4). He and his collaborators enzymatically cleaved the tobacco mosaic virus (TMV) coat protein and demonstrated that several of the peptide fragments were able to inhibit precipitation of the virus by rabbit antibodies to the virus. These results indicated that antibodies against the virus recognized various regions exposed on the surface of the TMV protein. It was shown subsequently that antiserum against an isolated tryptic peptide derived from the carboxy terminus of the TMV protein, when coupled to bovine serum albumin (BSA) as a carrier, gave rise to antibodies against the virus. Moreover, antibodies to a synthetic version of that same peptide were able to inactivate the infectivity of the virus (5,6). In a similar approach Fearney et al. (7) localized the antigenic determinants on the isolated TMV protein. They synthesized one of the determinants, a decapeptide corresponding to an internal region of the polypeptide chain, and raised antibodies against it which bound to native TMV protein but not, interestingly enough, to intact virus. The suggestion that this particular determinant is not exposed in the virion was later confirmed when the detailed fine structure of TMV was elucidated by X-ray diffraction (8). In 1976 Langbeheim et al. (9) localized an antigenic determinant on the coat protein of coliphage MS2. They went on to synthesize a peptide corresponding to this determinant that gave rise to neutralizing antibodies against the virus.

Studies by Arnon and Sela (10) and Arnon et al. (11) on the "loop peptide" of lysozyme emphasized the importance of conformation of antigenic determinants. The "loop peptide" consists of amino acid residues 60 to 83 of lysozyme; it contains a disulfide bridge between residues 64 and 80. Antisera against native lysozyme react with the isolated loop peptide. Vice versa, antisera raised against the "loop peptide," produced either synthetically or by enzymatic degradation, react with intact lysozyme. Antibodies against the "loop peptide" did not bind to the open chain peptide, however. Thus, it was concluded that the antigenic determinant contained in the "loop peptide" must be conformation-dependent.

It appeared from these and other studies that the production of antisera against native proteins when peptides are used as

immunogens required two conditions. First, the antigenic determinants in the protein had to be known, since it was presumed that only peptides corresponding to such determinants would be suitable. Second, the determinants of the native protein and the corresponding peptides used for immunization would have to have similar conformations. Presently, there are only a few proteins for which both of these prerequisites can be fulfilled. On the other hand, there is a rapidly increasing number of proteins whose amino acid sequences are available by inference from the nucleotide sequences of the corresponding genes, and it would be highly desirable if immunochemical procedures could be used in their identification and characterization.

Initially it appeared that raising antibodies against most of these "gene-characterized" proteins would be difficult, since procedures for their purification had not been worked out. Many of these proteins occur in very small quantities, either in cells or in extracellular fluids. Some have not even been identified yet, although they are known to exist on functional or genetic grounds. Moreover, until recently it seemed totally unrealistic to attempt the raising of antisera against peptide portions of these proteins if their antigenic and conformational properties were not known. This attitude is attested to by the complete absence of recorded attempts even though the technology existed.

The publication of the nucleotide sequence of the entire SV40 genome (12,13), from which the amino acid sequences of the SV40 large and small tumor antigens could be deduced, prompted us to ask whether antibodies could be raised against synthetic peptides corresponding to specific regions of these proteins. Since we knew neither the antigenic determinants nor the three-dimensional structure of these proteins, we chose regions for synthesis and immunization based on theoretical considerations alone (size, amino acid composition, location in the protein). Indeed, the antibodies produced were capable of reacting with native large T-antigen (Figure 1), demonstrating immediately that a prior knowledge of native antigenic determinants was unnecessary (14). Since then this approach has been used successfully for preparing antisera against a large number of proteins, many encoded by viruses. In the following paragraphs we summarize our experience in preparing antipeptide sera and describe various applications in the study of viral and other proteins. We also discuss the potential for synthetic peptides as vaccines.

NATURE OF ANTIGENIC DETERMINANTS

A very detailed and complete analysis of antigenic determinants has been carried out with sperm whale myoglobin and lysozyme (3,15,16), proteins whose amino acid sequences and

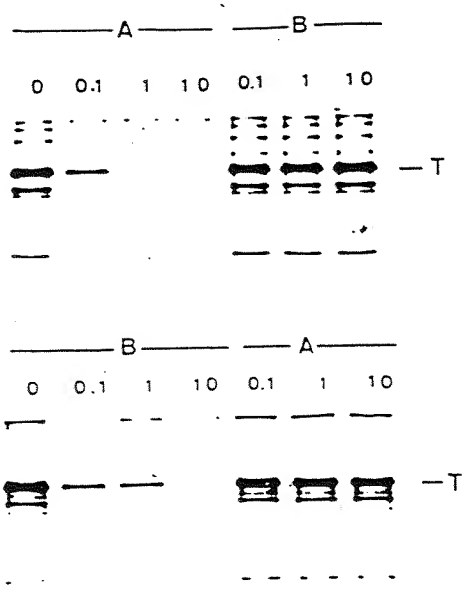

Figure 1. Immunoprecipitation of SV40 large T-antigen with antisera against synthetic peptides and inhibition of precipitation with synthetic peptides. Large T-antigen was immunoprecipitated from [^{35}S]methionine-labeled extracts of SV40-infected TC7 cells with antiserum to peptide A (upper panel) or to peptide B (lower panel) in the presence of various amounts (0.1-10 µg) of peptide A or B. Precipitates were analyzed on a 12.5% polyacrylamide gel. The lower portions of the gels do not contain radioactive bands and are not shown. Peptide A: AcNH-Met-Asp-Lys-Val-Leu-Asn-Arg-Tyr-COOH (amino terminus). Peptide B: NH_2-Lys-Pro-Pro-Thr-Pro-Pro-Pro-Glu-Pro-Glu-Thr-COOH (carboxy terminus). The tyrosine residue in peptide A is not part of T-antigen at this position. It was used to couple the peptide to BSA (from Ref. 14).

three-dimensional structures have been known for many years. In its native form myoglobin has five antigenic determinants, each of which consists of six to seven predominantly polar amino acids in a consecutive sequence. All are located on the outside of the molecule and usually, but not always, at bends between α-helical segments. Their length is approximately 20 Å, similar to that of the antigen-combining site on the immunoglobin molecule, which covers an area of 15 x 6 Å and is approximately 6 Å deep (17). It is remarkable that the same five determinants, with little or no variation in sequence on either side, are recognized in

various species (rabbit, goat, chicken, cat, pig, mouse). There is, however, a variation in the response of individual hosts of the same species (3). In contrast to myoglobin, the determinants in lysozyme are not continuous in sequence but consist of amino acids contributed from different parts of the polypeptide chain. Apparently all the natural determinants in myoglobin and lysozyme are conformation-dependent, i.e., antibodies against the native proteins do not recognize denatured proteins. In the case of lysozyme the antigenic structure is destroyed by oxidation of disulfide bridges (10,11).

Recently the four antigenic determinants of the influenza virus hemagglutinin were elucidated by amino acid sequencing, X-ray diffraction and the selection of variants by growth of the virus in the presence of monoclonal antibodies against hemagglutinin (18,19). Other proteins whose antigenic determinants have been studied include cytochrome c (20,21), TMV protein, serum albumin (22), human hemoglobin (23-25), human IgG (26), ferredoxin from Clostridium pasteurianum (27), myelin basic protein (28,29), bovine α-lactalbumin (30), hepatitis B surface antigen (31), structural protein VP1 of foot and mouth disease virus (32), Streptococcus pyogenes M protein (33) and diphtheria toxin (34). Some of these proteins will be discussed in more detail below (see section on synthetic vaccines).

CHOOSING SEQUENCES FOR SYNTHESIS AS PEPTIDE ANTIGENS

Given the complete or partial amino acid sequence of a protein, the main goal will be to select a region from this protein that is immunogenic when coupled to a carrier and that will give rise to antibodies that recognize the intact native and/or denatured protein. Usually the selection must be successful in the absence of any experimental knowledge of the antigenic or three-dimensional structure of the protein. Consideration must be given, also, to the ultimate goal of the study. If the intent is to identify a protein by SDS-polyacrylamide gel electrophoresis (PAGE) of an immunoprecipitate, then it is enough to select a region which is likely to be exposed in the isolated protein (although it is possible to use denatured proteins in these circumstances). If the intent is to neutralize a virus, then the segment chosen must be also exposed in the intact virus. And if the goal is to block some physiologic function attributable to that protein, the segment must be near to or at the active or functional site of the protein. In all these cases it is reasonable to begin by selecting segments that are likely to be exposed in the individual protein. As such, peptide sequences that have substantial polar character will ordinarily be the segments of choice. This view is based on the longstanding notion that the interiors of proteins are

predominantly hydrophobic, hydrophilic sidechains, and especially charged groups, being restricted to the outer shell of a protein where they have access to the aqueous environment (35).

Amino- and Carboxy-Terminal Sequences

More often than not, a suitable segment can be selected by careful "visual" examination of the amino acid sequence (i.e., without recourse to a computer scan). The carboxy terminus is exposed in many proteins, usually having a negatively charged carboxyl group. Moreover, carboxy-terminal peptides can be attached to carriers and supports via their α-amino groups, thereby simulating their natural appearance (14). For much the same reasons, amino-terminal peptides are also good choices, although in this case, one has to be alert to the possibility of various post-translational modifications, including the removal of initiating methionine residues or the acetylation of α-amino groups, especially in uncharacterized proteins known only through their DNA sequences. The modified peptide could have a quite different character from the unmodified one. In many cases where the nature of the amino-terminus is not known, it is reasonable to split the synthetic peptide into two portions, acetylating one in the event the native material is acylated, thus covering one common modification. In any event, amino-terminal peptides are usually attached by their carboxy-terminal residue.

Internal Sequences and Computer Scanning

Polar sequences that occur other than at the amino- or carboxy-termini can also be "spotted by eye," but the identification of those segments most likely to protrude is greatly facilitated by computer analysis. A number of reports describing suitable computer programs have already appeared. In essence, they are all similar. A polarity scale for the 20 amino acid side chains is devised and the sequence of a protein progressively evaluated as a moving average, the span of which is usually set between 5 to 9 residues. The resulting graph shows the most pronounced polar and nonpolar sectors as peaks and valleys (or vice-versa) (Figure 2).

Rose (36) and Rose and Roy (37) were the first to show that such an approach could distinguish interior regions from exterior ones. At about the same time, Both and Sleigh (38), employing a program devised by A. Reisner, compared the relative hydrophobicities of the hemagglutinins of human influenza virus and fowl plague virus. These authors were interested in changes in viral antigenicity in the face of evolutionary change, on the one hand, and a possible lack of antigenicity in structurally conserved regions, on the other. Hopp and Woods (30), using as a

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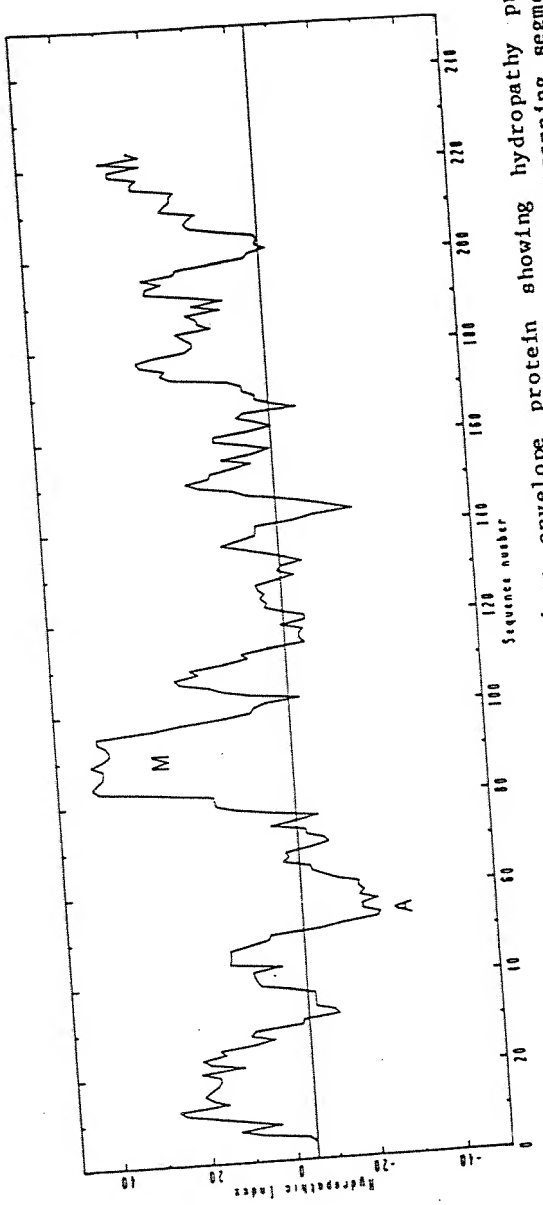


Figure 2. Computer plot of hepatitis B antigen envelope protein showing hydropathy profile according to scheme of Kyte and Doolittle (Ref. 40). M is likely a membrane-spanning segment; A and B are hydrophilic sections that might be considered for synthesis as peptide immunoprobes.

guide for hydrophilicity a scale devised by Levitt (39), evaluated a dozen proteins with known antigenic determinants in an effort to correlate hydrophilic regions with antigenicity. Kyte and Doolittle (40) devised a scale that takes into account both hydrophobicity and hydrophilicity, using a property they called hydropathy (Gr: "feeling for water") (Figure 2).

The study by Hopp and Woods (30) asserts that not all antigenic determinants are associated with peaks of hydrophilicity and, conversely, not all hydrophilic regions necessarily represent antigenic determinants. This is not to say that the latter regions (hydrophilic but not antigenic in the native protein) are not suitable for raising antibodies. In fact, the carboxy-terminal region (eleven amino acids) of SV40 large T-antigen appears not to be immunogenic in the native protein. When used for immunization, however, the same region in the form of a peptide-BSA conjugate induces antibodies that readily recognize the carboxy terminus in native large T (41). Thus, choosing a region for synthesis and immunization that is immunogenic in the native protein is not a prerequisite for the success of the peptide approach.

Secondary Structure Predictions

In addition to polarity, considerations of secondary structure can be helpful for selecting suitable sequences for synthesis. Thus, "β-turns" frequently exist in proteins as elbows jutting out from the main body. Chou and Fasman (42,43) have shown that the amino acid residues most frequently occurring in turns are asparagine, aspartic acid, proline and glycine, proline most often occupying the second position from the amino-terminal end of the turn, as in Asn-Pro-Gly-X. Accordingly, Hopp and Woods (30) increased the "hydrophilic value" of proline in their computer program. Pfaff and collaborators, in an evaluation of the antigenicity of foot-and-mouth disease virus capsid proteins, also considered the "sidedness" of α-helices, the helical wheel approach of Schiffer and Edmundson (44) being used as a guide for finding exposed segments (44a).

It should be pointed out that none of these programs is suitable for dealing with the conformation problem mentioned earlier. Clearly, antibodies recognize conformations that result from sequence and not sequence per se. For example, antisera prepared against fragments of ribonuclease react well with the fragments themselves but poorly with the native enzyme. Conversely, antibodies prepared against native ribonuclease react only weakly with "unfolded" fragments. The extent of reaction increases with increasing concentration of fragments, however, and it has been postulated that the random, unfolded forms of the fragments are in equilibrium with fragments in a native

configuration (45). The latter, representing a very small fraction of the total, probably bind to the antibodies raised against the native protein. Similar considerations could explain why antisera against small peptides are capable of reacting with the native proteins from which they are derived. Peptides may exist in solution as a mixture of many different conformations that are in equilibrium. One of these may resemble the corresponding region in the native protein and, if recognized by the immune system, may give rise to antibodies capable of reacting with the native protein. The majority of conformations, however, may induce non-crossreacting antibodies. It is also possible that antibodies against conformations that are similar to but not identical with the native conformation at first bind weakly to the native protein and then cause a change in conformation of the antigenic site, resulting in strong binding. Antibody-induced conformational changes have been described (46,47). We have estimated that the fraction of antibodies in antipeptide sera capable of reacting with the native protein may be of the order of 0.1 to 1%, as one might expect given the above considerations. This fraction is usually sufficient, however, and the affinity of the antibodies for the native proteins is high enough for most studies.

ATTACHMENT TO CARRIERS

At the time a segment is being selected for synthesis, attention should also be given to the intended mode of attachment, since the particular nature of the sequence can exclude certain attachment schemes. The reader should consult the rich source of attachment procedures compiled in a book edited by Jaffe and Behrman (48). Four commonly used schemes are:

a) glutaraldehyde for amino to amino coupling (49,14)
b) water-soluble carbodiimides for carboxyl to amino coupling (50)
c) bis-diazobenzidine (BDB) for tyrosine to tyrosine sidechain coupling (51,14), and
d) maleimidobenzoyl-N-hydroxysuccinimide ester for coupling cysteine (or other) sulfhydryls to amino groups (52,53).

Each reagent has advantages and disadvantages. The general rule as far as the selection of sequences is concerned is: the group involved in attachment should occur only once in the sequence, preferably at the appropriate end of the segment. For example, BDB should not be used if a tyrosine residue occurs in the main part of a sequence chosen for its potentially antigenic character. Similarly, centrally located lysines rule out the glutaraldehyde method, and the occurrences of aspartic and glutamic acids frequently exclude the carbodiimide approach. On

the other hand, suitable residues can be positioned at either end of a chosen sequence segment as attachment sites, whether or not they occur in the "native" protein sequence (14).

Internal segments, unlike the amino and carboxy termini, will differ significantly at the "unattached end" from the same sequence as it is found in the native protein where the polypeptide backbone is continuous. The problem can be remedied, to a degree, by acetylating the α-amino group and then attaching the peptide by way of its carboxy terminus.

The coupling efficiency to the carrier protein is conveniently measured by using a radioactively labeled peptide, prepared either by using a radioactive amino acid for one step of the synthesis or by labeling the completed peptide by the iodination of a tyrosine residue. The presence of tyrosine in the peptide also allows one to set up a sensitive radioimmune assay, if desirable. Therefore, we often introduce tyrosine as a terminal residue if it is not part of the determinant already. We have used BSA as a carrier in all cases, but others have used keyhole limpet hemocyanin (KLH) with good success (31,53-56). We prefer BSA because peptide-BSA conjugates are usually soluble whereas KLH has a tendency to precipitate during coupling. This makes KLH less convenient to handle, although it may actually increase its immunogenicity. In our experiments the molar ratios of peptide to BSA in various conjugates have ranged from 4 to 30.

IMMUNIZATION

The number of known effective immunization regimens is enormous (48). They differ in the amount of antigen used for the primary injection and subsequent boosts, the route of injection, the time schedule for injecting and bleeding, and the use of incomplete or complete Freund's adjuvant. It would be very time consuming to check out all parameters, and we have settled for a scheme that uses a high dose of conjugate, complete Freund's adjuvant and intradermal injections at 20 different sites of the rabbit for the primary injection and subcutaneous and intramuscular injections for boosting in four-week intervals. Positive serum is usually obtained four weeks after the primary injection. In some cases the titers do not increase following boosting, whereas in others it may increase by a factor of 2 to 5. In several instances antibody titers dropped drastically if boosting was discontinued for 3 to 4 months and did not increase again following additional boosts (G. Grob and G. Walter, unpublished). We do not understand this phenomenon. Individual rabbits may be poor responders. Accordingly we prefer to start immunizing three to four rabbits with one peptide and then keep only the good responders for collecting serum. It is recommended that boosting be continued over a period of 4 to 5 months since antibody production can increase during this period of time.

ASSAYS FOR ANTIBODIES

A fast, sensitive and quantitative assay for testing antipeptide sera is an enzyme-linked immunosorbant assay (ELISA). Because the assay is quantitative and quick, it is highly recommended for screening individual bleeds from various immunized rabbits. Because of the extreme usefulness of this assay we describe it here in some detail. It is based on the method of Voller (57) and was adapted for testing antipeptide sera (H. MacArthur, R. Friis and G. Walter, unpublished). As in other solid phase immunoassays, the test is based on the tendency of macromolecules to adsorb nonspecifically to plastic. The irreversibility of this reaction, without loss of immunological activity, allows the formation of antigen-antibody complexes with a simple separation of such complexes from unbound material.

To titrate antipeptide serum, peptide conjugated to a carrier different from that used in immunization is adsorbed to the wells of a 96-well microtiter plate. The adsorbed antigen is then allowed to react in the wells with dilutions of anti-peptide serum. Unbound antibody is washed away, and the remaining antigen-antibody complexes are allowed to react with antibody specific for the IgG of the immunized animal. This second antibody is conjugated to an enzyme such as alkaline phosphatase. A visible colored reaction product produced when the enzyme substrate is added indicates which wells have bound antipeptide antibodies. The use of spectrophotometer readings allows better quantification of the amount of peptide-specific antibody bound. High-titer antisera yield a linear titration curve between 10^{-3} and 10^{-5} dilutions.

In many cases the goal is to identify a viral or cellular protein with antipeptide serum. The most direct way for doing this is immune precipitation and analysis of the precipitate by PAGE. As an example, we usually test 10 and 100 µl of crude serum with 100 µl of radioactive cell extract from 1 to 2 x 10^5 cells. As controls we use either preimmune serum or immune serum in combination with a large excess of the peptide used for immunization. The peptide competitively inhibits precipitation of specific proteins but not of nonspecific background. As such, this control proved invaluable when the protein to be identified represents only 0.1% or less of the total cellular proteins. The acrylamide gel will reveal numerous nonspecific proteins, many of which are more intense than the specific protein (58) (Figure 3). To reduce background and achieve identification of a particular protein, it may be necessary to purify peptide-specific antibodies by affinity chromatography on peptide covalently coupled to Sepharose (58).

Antipeptide sera are also useful for the intracellular localization of proteins by immunofluorescence. Positive titers of 1:50 to 1:2000 were obtained for an antiserum to the carboxy

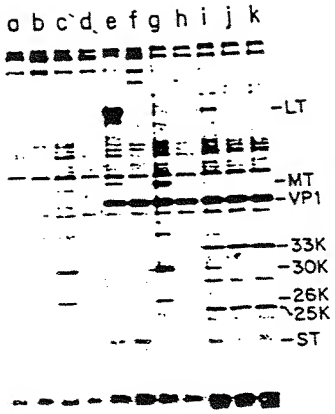


Figure 3. Immunoprecipitation of polyoma virus medium T-antigen and crossreacting cellular proteins with antiserum against the carboxy-terminal peptide of medium T-antigen, Lys-Arg-Ser-Arg-His-Phe. Mouse 3T6 cells were mock-infected (lanes a-d) or infected with the polyoma deletion mutant dl-8 (lanes e-k), and radiolabeled 24-27 hr later. Extracts were precipitated with: 10 µl of rat antitumor serum (lanes a and e), 10 µl of preimmune rat serum (lanes b and f), 4 µl of affinity purified antipeptide serum (c and g), 4 µl of control serum (the flow-through from the affinity column) (lanes d and h), or 4 µl of antipeptide serum in the presence of 0.1, 1, and 10 µg of peptide (lanes i, j, and k). LT, large T-antigen; MT, middle T-antigen; ST, small T-antigen. Numbers are molecular mass in kilodaltons (from Ref. 58).

terminus of SV40 large T-antigen. Again, as in immunoprecipitations, the specific fluorescence is inhibitable by the peptide used to raise the antibodies. For unknown reasons, basic peptides may be prone to artifact in cellular settings (T. Hunter, personal communication).

Since it is the nature of antipeptide sera to recognize sequence-specific rather than conformation-specific determinants, it is perhaps not surprising that they bind to SDS-denatured proteins. Accordingly, antipeptide sera may also be used for detecting specific proteins by "Western blotting" (59). In this technique proteins are electrophoretically transferred from SDS-polyacrylamide gels onto nitrocellulose paper. Individual proteins may then be detected by reaction with an antipeptide serum. For example, if a crude extract of SV40 infected monkey cells is fractionated by PAGE and transferred to nitrocellulose paper, large T-antigen can be easily detected with an antiserum against the carboxy-terminal peptide (G.W. and B. Dietrich, unpublished observation). In this context it is worth mentioning that antiserum prepared against purified SDS-denatured SV40 large

T-antigen reacts with native and denatured large T, whereas antiserum against native large T-antigen (SV40 tumor serum) only reacts with the native protein. The transforming protein of Rous sarcoma virus, p60src, is detectable on Western blots with antiserum to the carboxy-terminal hexapeptide of p60src (B. Sefton and E. Nigg, personal communication). On the other hand, we were unable to detect polyoma virus medium T-antigen by Western blotting with antiserum against the carboxy terminus of medium T. The reasons for this are not known.

THE PROBLEM OF CROSSREACTIVITY

Antisera against peptides may crossreact with other proteins, related and unrelated to the protein under study. This problem was recognized and aptly described by Anderer and Schlumberger as early as 1965 (5). They observed that antisera against the carboxy-terminal hexapeptide region of the TMV vulgare structural protein crossreacted with the structural protein of TMV Dahlemense, whose carboxy-terminal sequence differs from that of strain vulgare in two positions:

vulgare	Thr-Ser-Gly-Pro-Ala-Thr
Dahlemense	Thr-Ser-Ala-Pro-Ala-Ser

Furthermore, antisera against the carboxy-terminal penta-, tetra-, tri- and dipeptides of strain vulgare also crossreacted with proteins from Dahlemense. A correlation between peptide size and crossreactivity was observed in that antisera against shorter peptides crossreact more strongly than those against longer ones. Crossreaction was tested both by precipitation reaction and neutralization of infectivity. In all cases the reaction of antisera with the homologous virus (strain vulgare) was stronger. The immune reactions were inhibited by peptide, ruling out artifacts such as nonspecific adsorption. Interestingly, antisera against longer peptides required more peptide for inhibition than antisera against shorter peptides, indicating that the antibodies against the longer peptides had a higher affinity. Surprisingly, antiserum against threonine alone, the carboxy-terminal residue, coupled to BSA, was capable of inactivating TMV vulgare. This serum had no effect on TMV Dahlemense, which has carboxy-terminal serine (6).

Recently, similar crossreactions have been observed with other antipeptide sera. Antibodies against the amino-terminal heptapeptide of SV40 large T-antigen crossreact weakly with polyoma virus large T-antigen whose amino-terminal sequence differs at two positions (60):

SV40	Ac-Met-Asp-Lys-Val-Leu-Asn-Arg
Polyoma	Ac-Met-Asp-Arg-Val-Leu-Ser-Arg

On another front, antiserum against the carboxy-terminal hexapeptide of polyoma virus medium T-antigen cross-reacts with two cellular proteins from uninfected mouse cells (mw 30,000 and 26,000) (Figure 3). In another case, antiserum against the carboxy-terminal hexapeptide sequence of p60src of RVS, when used at low concentration, is specific for p60src both in immunoprecipitation and by immunofluorescent microscopy and crossreacts only weakly with two cellular phosphoproteins (61). At high concentrations, however, additional crossreaction with three cytoskeletal proteins, myosin, tubulin and vimentin, was observed (62). In all cases the observed crossreactions are specific in the sense that they can be inhibited completely with the corresponding peptides (Figure 3).

What is the biological significance of the observed crossreactions? Although it is tempting to speculate about functional and evolutionary relationships between crossreacting proteins, there is no evidence to suggest that such relationships exist in all cases mentioned above. Indeed, crossreactivity has been found serendipitously between totally unrelated proteins (62a).

Crossreactivities have also been observed with monoclonal antibodies, although the functional significance in these instances has been questioned (63-65a). When a series of monoclonal antibodies directed against SV40 large T-antigen was isolated, for example, several reacted with proteins in uninfected cells (64). In no case was the same host protein recognized by two (or more) antibodies of different specificity. It is possible, or even likely, that all these crossreactions are fortuitous. Crawford et al. (64) made an attempt to estimate the expected frequency of random crossreaction and concluded that the frequency depends on the number of residues involved in a given epitope. Thus, they estimated that an antibody recognizing a sequence of four or fewer amino acids was virtually certain to encounter an identical sequence in another protein in almost any cell, but the odds against a heptapeptide match were of the order of 250 to 1 (64).

Another interesting observation made by Crawford et al. (64) was that all crossreacting monoclonal antibodies were directed against denaturation-insensitive determinants, whereas none of the antibodies directed against denaturation-sensitive determinants crossreacted with other proteins. Since antipeptide sera are likely to be directed against denaturation-insensitive (sequence-specific) determinants, the questions and problems related to crossreactivity are of a similar nature for both monoclonal antibodies and antibodies to synthetic peptides. Another point to be considered is that immunization with a six to ten amino acid peptide most likely elicits an immune response against a large number of determinants located on overlapping segments of the peptide or made up of different conformations of the peptide or segments thereof. This factor may increase the

chances for crossreaction. At present our knowledge of antibody-antigen interactions on a molecular scale is still quite limited. In particular we do not understand the relative contributions of conformation and sequence to antigenicity. Generally speaking, it appears that as conformation becomes a more important factor, the chances for crossreaction decrease.

Despite the potential problems arising from crossreactions, in our experience antibodies against synthetic peptides have always preferentially precipitated those proteins from which the peptide sequences were derived, and the number of specifically crossreactive proteins was always small (see "Discovering New Gene Products").

PROTEIN PURIFICATION

Antipeptide antibodies in combination with the corresponding peptides can be used for purification of proteins by affinity chromatography. For example, we have used antibodies against the synthetic peptide Lys-Arg-Ser-Arg-His-Phe, corresponding to the six carboxy-terminal amino acids of the polyoma virus medium T-antigen (58), to purify medium T from infected cell extracts (66). The purification procedure is as follows. First, the IgG fraction of the antipeptide serum prepared from total immunoglobulins by affinity chromatography on a peptide-Sepharose column, is coupled to Sepharose. Then, an extract of polyoma virus-infected cells is incubated with the antipeptide Sepharose to allow binding of the medium T-antigen to the antibody, after which proteins bound non-specifically are removed by washing. Finally, the specifically bound medium T-antigen is eluted with an excess of the peptide. Figure 4 illustrates the result of a typical binding and release experiment.

This purification procedure has several advantages:

1. The elution from the immunoaffinity column is accomplished by the use of an excess of peptide in isotonic salt at neutral pH rather than by chaotropic agents or extremes of pH. In general this should yield proteins in a functional state, which may not always be the case where denaturing conditions have been used for elution.
2. Substantial purification can be achieved in a single step in a short time (2,500 fold in 4 hr in the case of medium T-antigen). This will be a benefit where labile proteins are being examined.
3. Unlike conventional methods, such purifications of proteins can be achieved on a small scale. For instance, only 10^5 cells were used in the purification of medium T-antigen. It should also be possible to use

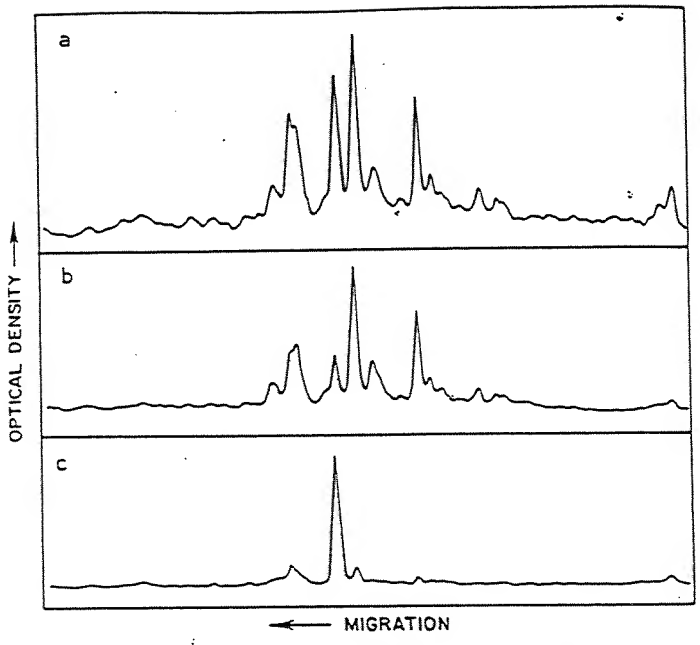
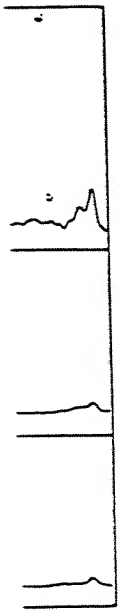


Figure 4. Purification of polyoma virus medium T-antigen by affinity chromatography. [^{35}S]Methionine-labeled extracts from 10^5 mouse 3T6 cells infected with the polyoma virus deletion mutant dl-8 were immunoprecipitated with 25 µl of antipeptide-Sepharose. Specifically bound proteins (a), proteins eluted by exposure to 10 µg of peptide in 50 µl of RIPA buffer containing 1 mM dithiothreitol (c), and proteins remaining after elution with peptide (b) were analyzed by gel electrophoresis. The peaks corresponding to the medium T-antigen are shaded in each case (from Ref. 66).

the method to prepare large amounts of proteins by increasing the capacity of the column.

4. The technique works well for the purification of proteins present in low concentrations in extracts. In general such proteins are difficult to purify by conventional means.
5. Since the procedure is fast and simple it can be used in situations where multiple samples are being analyzed as, for example, when wild type and mutant proteins are to be compared under various conditions. Another useful feature of this approach is that it can be used sequentially. In principle, a protein purified through the use of one such immunoaffinity absorbent can be purified further by use of a second antiserum directed against another region of the same protein in combination with



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the cognate peptide. In this way it should be possible to eliminate crossreacting proteins still present after the first purification.

In the case of medium T-antigen we found that a non-denaturing mixture of detergents was required in addition to peptide for efficient elution of the protein from the antipeptide Sepharose (66). This is puzzling because the initial binding of the medium T-antigen to the antipeptide Sepharose can be prevented by peptide alone, and in the absence of detergents. One possible explanation is that after initial binding of the medium T-antigen to the antipeptide Sepharose secondary binding reactions occur which can only be reversed in the presence of detergents. In the case of medium T-antigen the hydrophobic region of 22 amino acids adjacent to the carboxy terminus may be responsible for a secondary interaction with the antibody. In other cases detergents are not required for release. For example the SV40 large T-antigen, bound to Sepharose by antibodies directed against a carboxy-terminal peptide, can be eluted by the peptide alone (G. Grob and G. Walter, unpublished observations). It is clear, therefore, that the conditions for the release of a protein bound to an antipeptide antibody will vary depending on the nature of both the protein and the peptide and will have to be optimized in each instance. In one instance, when SV40 large T-antigen was bound to antibodies against a peptide corresponding to the amino-terminal eight amino acids, we have been unable to elute the protein with the peptide under any conditions tried (G. Grob and G. Walter, unpublished).

SUBCELLULAR LOCALIZATION OF PROTEINS

Antisera against synthetic peptides can be used to determine the subcellular localization of a viral or cellular protein by indirect immunofluorescence microscopy. This approach has been successfully used for SV40 large T-antigen (41) and p60src of Rous sarcoma virus (Figure 5). Since the location of the antigenic determinants recognized by antipeptide sera is known, specific statements about the arrangement or orientation of a protein in a particular cell compartment (for example, plasma membrane) can be made (67). In this respect, antipeptide sera have an advantage over conventional polyclonal or even monoclonal antibodies for which the location of determinants recognized is usually unknown. For example, with antiserum against the carboxy terminus of SV40 large T-antigen it was shown that the carboxy terminus of this protein is exposed on the surface of SV40 transformed cells (41). In general, by using antibodies against various regions of a membrane protein its topography on either side of the membrane can be delineated. Because of potential

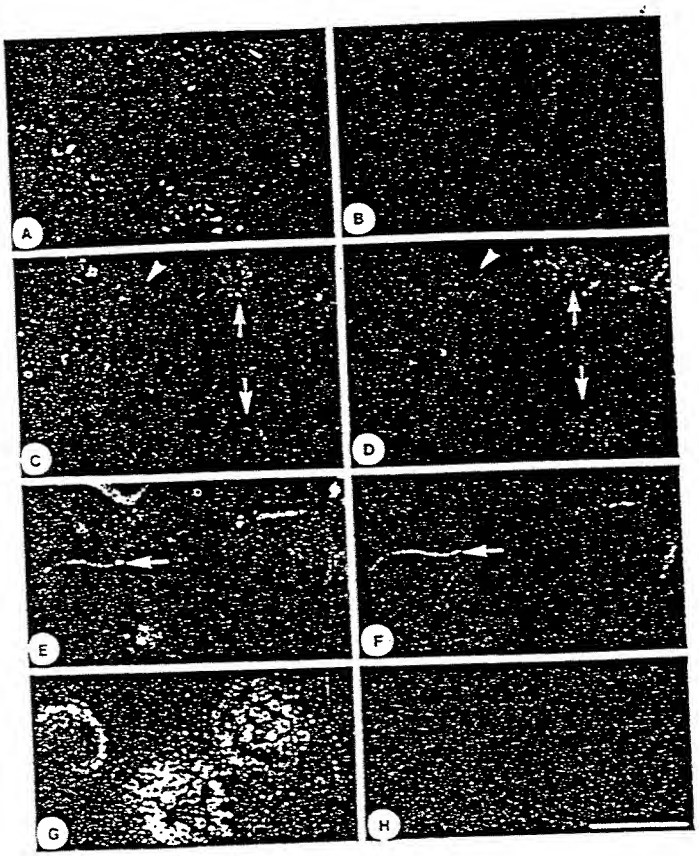

Figure 5. Immunofluorescent localization of the transforming protein of Rous sarcoma virus with antibodies against a synthetic src peptide. Double indirect immunofluorescent labeling of normal rat kidney cells (A and B) and B77 transformed rat kidney cells (C-H). The cells in A, C, E, and G were immunolabeled for vinculin; the same cells were immunolabeled for p60src by using anti-src-c IgG in B, D, F, and H, respectively. Affinity-purified guinea pig antibody to chicken gizzard vinculin was used at 40 µg/ml; affinity-purified rabbit anti-src-c IgG was used at 2 µg/ml. In G and H, soluble src-c peptide at 50 µg/ml was incubated with the mixed primary antibodies. Secondary antibodies were fluorescein-conjugated affinity-purified goat antibodies to guinea pig IgG and rhodamine-conjugated affinity-purified goat antibodies to rabbit IgG. The microscope focused at the level of the substrate in A-D, G, and H or at mid-cell level in E and F. (Bar in H represents 20 µm) (from Ref. 67).

crossreactivity problems, however, caution must be exercised in the use of antipeptide sera in immunofluorescent microscopy (62).

DISCOVERING NEW GENE PRODUCTS

Antibodies to synthetic peptides provide a novel approach for the identification, isolation and characterization of unknown proteins whose genes have been isolated and sequenced (14). One such example is the identification of the transforming protein of Moloney murine sarcoma virus (MuSV) (68, 69). The gene encoding this protein had been sequenced (70, 71) and shortly thereafter antisera were produced against a synthetic peptide based on its predicted carboxy terminus. The antisera were able to precipitate a protein of 37 K, corresponding to the transforming gene. The protein represents only 0.0002 to 0.0008% of the total cellular protein in the MuSV-transformed cells (10,000 to 40,000 molecules per cell) and was detectable on polyacrylamide gels as a minor band which could only be distinguished from many background proteins by specific inhibition with excess peptide (69). Previous attempts to immunoprecipitate this protein with conventional tumor sera had been unsuccessful.

Antibodies have been produced against synthetic peptides corresponding to the carboxy-terminal sequence of the polio virus genome-linked protein VPg (72, 73). These antibodies, in addition to precipitating VPg, precipitate precursor proteins containing the sequence of VPg at their carboxy terminus. In another case, a hypothetical protein from human mitochondria, URF6L, has been identified on the basis of precipitation by antibodies raised against a peptide corresponding to its theoretical carboxy terminus (Mariottini et al., in preparation).

A large number of viral and cellular gene products await identification in the future. In many cases these proteins will be minor components and therefore difficult to identify and to purify. In such circumstances the "peptide approach" is clearly the method of choice. Still, care must be taken in making judgments as shown by one study where a supposed new gene product encoded by a mouse leukemia virus (Mo-MuLV) was precipitated by antipeptide serum but turned out later to be a known structural protein (p15E) of the virus (74, 75).

SYNTHETIC PEPTIDES AS VACCINES

The drawbacks of presently used vaccines and the future prospects of synthetic vaccines have been discussed by others (76-80). In the following section we outline only the most recent developments. It is fair to say that the "dream of synthetic vaccines" (79) has drawn somewhat closer to reality.

Foot-and-Mouth Disease Virus (FMDV)

Foot-and-mouth disease virus is a positive-strand RNA virus of the picornavirus family whose capsid is made of four proteins, VP1 to VP4. As far as is known, neutralizing antibodies against FMDV are induced by VP1 only. In a recent study various defined fragments of VP1, obtained by cleavage with cyanogen bromide and proteolytic enzymes, were tested for their ability to induce neutralizing antibodies to the virus (32). Two regions of VP1, one between amino acids 138 and 154, the other between 200 and the carboxy terminus, were found to be crucial for raising neutralizing antibodies. The amino acid sequences of VP1 from two strains of type A and one of type O have been deduced from nucleic acid sequences (81-83). In all cases, the carboxy terminus can be cleaved off without loss of infectivity whereas cleavage within the region from 138 to 154 destroys infectivity. It is assumed that the latter part plays a role in attachment of the virus to the cell (32).

Making use of this information, two groups have attempted to raise neutralizing antibodies against FMDV with synthetic peptides as immunogens. Bittle et al. (56) immunized with peptides corresponding to the amino- and carboxy-terminal regions of VP1 and one from the central region (residues 141 to 160). The choice of these regions was based on amino acid sequence comparisons between VP1 of different virus strains, which show an increased variability in the middle and carboxy-terminal but not the amino-terminal region. Therefore, the middle and carboxy-terminal regions might be targets of the immune system and subject to change under selective pressure. Virus-neutralizing activity was obtained with peptide 141 to 160 and peptide 190 to 213 (carboxy terminus) but not with amino-terminal peptides. Immunization with peptide 141 to 160 also resulted in protection of guinea pigs against viral infection. The authors claim that the protection is 10% of that obtained with inactivated virus and orders of magnitude better than that obtained with isolated VP1. This is in contrast to results obtained by Kleid and collaborators (82) who claim that genetically engineered VP1 (2×10^6 molecules per bacterium) is as good as peptide in protection experiments. For obvious reasons controversies in this area of research are not always dictated merely by scientific aspects (84,85). It is interesting that antiserum to peptide 141 to 160 does not react with VP1 on Western blots, possibly indicating that the peptide assumes a conformation which is different from the corresponding region in SDS-denatured VP1. Possibly this peptide assumes a conformation more similar to the corresponding region of VP1 present in intact virus than in SDS-denatured VP1 (56). This would also explain why antiserum against SDS-denatured VP1 shows little if any neutralizing activity.

Pfaff and co-workers used the helical wheel presentation (44a) to select a region from amino acid 144 to 159 of VP1 as a potential immunogenic site (E. Pfaff, personal communication). They successfully produced virus-neutralizing antibodies against a synthetic peptide corresponding to this region. Interestingly, almost all virus-neutralizing activity in antiserum against virus can be adsorbed out by the peptide, indicating that this region represents the dominant immunogenic site on the viral surface. These investigators point out that region 144 to 159 was not particularly obvious as a potential immunogenic determinant when the amino acid sequence of VP1 was analyzed by the Hopp and Woods (30) program, whereas it was obvious in the helical wheel presentation of Schiffer and Edmundson (44).

Polio Virus

It is remarkable that the immunogenic structure of polio virus is still unknown despite the fact that polio virus vaccines have been in use for over two decades (86,87). It appears that the epitopes responsible for inducing neutralizing antibody are extremely conformation-sensitive and dependent on the structure of intact virions. Even empty viral capsids elicit only a weak immune response. Recently, two hybridomas producing neutralizing antibodies against polio virus have been described. It was shown by crosslinking experiments that these antibodies bind to different sites on VP1 in intact virions (88). Variants of polio virus, not neutralized by these monoclonal antibodies, arise with high frequency during propagation of the virus. This finding indicates that the sites recognized by these two monoclonal antibodies are important immunogenic determinants with respect to virus-neutralization. One can expect that these studies will lead to a better understanding of the immunogenic structure of polio virus in the near future.

Influenza Virus

The three-dimensional structure of influenza virus hemagglutinin (HA), the major protein on the viral surface, has been elucidated in great detail by X-ray diffraction, and the locations of four antigenic sites on the HA molecule have been determined (18,19,89). Amino acid substitutions at these sites caused by mutation lead to the development of new viral strains with changed immunogenic properties. Site A (amino acids 140 to 146) is a loop of seven amino acids protruding from the surface of the molecule. Site B (amino acids 187 to 196) is α-helical and involved in binding of the virus to the cell receptor. Site C is located close to a disulfide bridge involving Cys 52 and Cys 278. Several non-contiguous amino acids contribute to this

site. Site D embraces amino acids 207 to 220; this site is unusual in that mutations (amino acid substitutions) affecting its interaction with antibodies are located outside of the site itself. As pointed out by Wiley et al. (19), it is not altogether understood why only these regions are immunogenic whereas others are not, although their prominence in the SOAP profile of Kyte and Doolittle is noteworthy (Figure 6).

Efforts to raise antibodies against HA have been made in three laboratories. Green et al. (55) used 20 synthetic peptides covering 75% of the HA polypeptide sequence. All peptides were immunogenic and most reacted weakly with HA or virus. It is not apparent from this study to what extent and by which criteria the antisera affect virus infectivity. It is interesting, although not unexpected, that none of the synthetic peptides was recognized by antiserum directed against whole virus, indicating that the conformations of the peptides and their corresponding regions in the protein are very different. It also emphasizes the point made earlier that antibodies directed against native proteins (conformational determinants) show less crossreaction than those against peptides (sequential determinants). One would have expected that peptides corresponding to those regions in the HA molecule where "natural" immunogenic determinants are located (sites A to D) would elicit a better and more specific response to HA and virus than antibodies against other regions. Such was not the case, however; on the contrary, antisera against several peptides covering site A, the protruding loop, did not react with HA or virus (55).

Jackson et al. (90) also synthesized a peptide (amino acids 123 to 151) covering the "loop" region (residue 140-146) and raised antibodies against it. No binding of the antipeptide antibodies to the virus was observed, however. There was some crossreaction between virus and peptide with antibodies against the virus, in contrast to the study by Green et al. (55). In a study by Müller et al. (91) a peptide corresponding to residues 91 to 108 of HA was synthesized and used for raising antibodies. Although this peptide does not overlap with any of the four antigenic sites in the native protein, it elicits antibodies in mice that confer partial protection against a challenge with mouse-adapted influenza virus. The authors point out the possible advantage of using a peptide corresponding to a conserved region of the HA molecule, avoiding the regions viruses may use to escape the immune system by antigenic drifting and shifting.

Hepatitis B Virus (HBV)

An estimated 200 million persons throughout the world are carriers of HBV, and 80,000 to 100,000 new cases occur in the U.S. each year, 10% of whom become chronic carriers of the

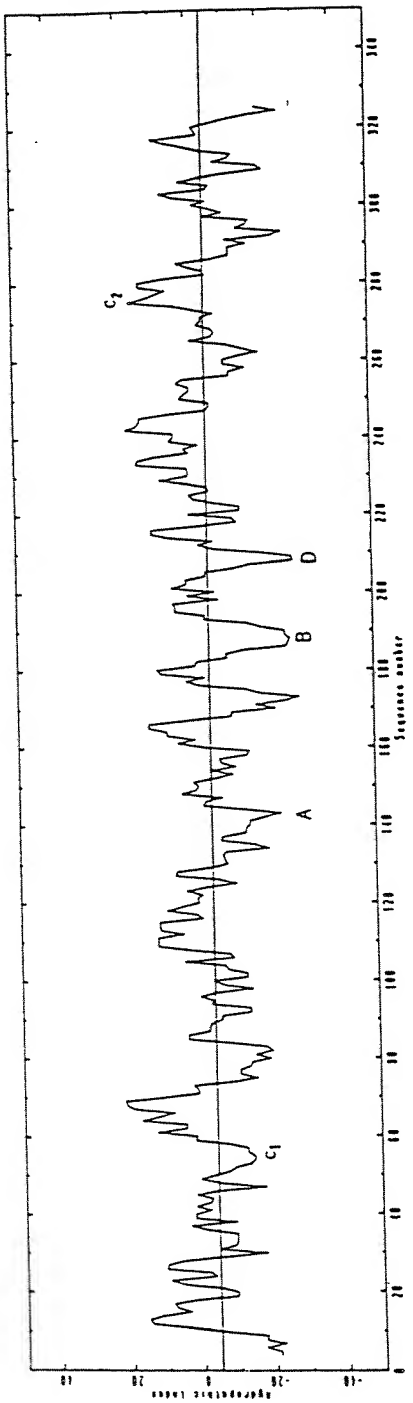

Figure 6. Computer plot of Influenza virus heavy chain (Hong Kong type) according to scheme of Kyte and Doolittle (Ref. 40). A-D correspond to antigenic sites identified by Wiley et al. (Ref. 19).

disease (92,93). The development of a conventional type of vaccine has not been possible because of the lack of a tissue culture system for the propagation of HBV. The only source for a vaccine has been the blood of HBV-carriers, which contains empty virus envelopes consisting of lipid and protein (93). A vaccine can be prepared by purifying the envelopes (92) or the major envelope protein (HBsAg, surface antigen) (94), which occurs in an unglycosylated (mw 25,000) and a glycosylated form (mw around 29,000). This type of vaccine has several drawbacks, including high cost and long procedures of purification and safety testing (65 weeks). Therefore, the development of a synthetic vaccine appears to be attractive, and several groups have initiated studies along this line. The 226-residue sequence of HBsAg was deduced from the nucleotide sequence of DNA (95-99). By Chou and Fasman analysis, only 4% of the molecule is α-helical (95). There are three hydrophobic regions occurring as β-pleated sheets, two at each end (residues 1 to 31 and 157 to 226) and one in the middle (residues 75 to 109). Two hydrophilic regions (residues 32 to 74, and 110 to 156) are located on either side of the hydrophobic region in the middle. It has been suggested that the three hydrophobic regions anchor the molecule in the plasma membrane and that the hydrophilic regions are exposed on the outside of the envelope and carry the immunogenic determinants (93). Various considerations and computer programs indicated that the most immunogenic region might be located around amino acid 145. Several peptides have been synthesized based on an analysis yielding the highest probability for β-turns between amino acids 142 to 145 (31,100). Prince et al. (101) and Hopp (102) used the computer program of Hopp and Woods (30) to locate the point of highest average hydrophilicity at residues 143 and 144. They synthesized a peptide corresponding to amino acids 138 to 149. The program of Kyte and Doolittle (40), which is similar to that of Hopp and Woods, was used by Lerner et al. (53) for the selection of several peptides from various regions throughout the molecule (Figure 2). Neurath et al. (54) synthesized the region corresponding to amino acids 135 to 155 based on the observation that chemical modification of lysine residues within this region resulted in loss of antigenicity. Finally, Dreesman et al. (103), on the basis of a high probability for turns and high hydrophilicity, chose the same general area and synthesized a peptide including amino acids 117 to 137. They also introduced a disulfide bridge between Cys 125 and Cys 137 in order to create a cyclic peptide.

The main results from the immunological studies of these synthetic peptides can be summarized as follows: 1. Most peptides were found to be immunogenic; only a few peptides failed to elicit an immune response (53). 2. Peptides corresponding to several regions throughout the HBsAg molecule can elicit antibodies against HBsAg. 3. Peptides including the amino acids 139 to 147 not only elicit antibodies against native HBsAg, but

in addition, this region is a major determinant in the native antigen (31). 4. The crossreactivity between peptides including region 139 to 147 and native HBsAg is weak. The following equilibrium constants have been measured: 1.4×10^8 for the reaction of HBsAg with anti-HBsAg, 3.8×10^7 for the reaction P 135-155 (peptide including amino acids 135 to 155) with anti P 135-155, and 4.3×10^5 for the reaction HBsAg with anti P 135-155 (54).

A computer prediction of the HBsAg structure reveals two prominent hydrophilic regions that, in theory, might be suitable for synthetic vaccines ("A" and "B" in Figure 2). Since the region labeled "B" (res. 140 to 145) is known to be the major antigenic determinant in the native protein (31), it must be exposed. If the region labeled "M" is a membrane-spanning segment, then the hydrophilic region denoted "A" would be located on the other side of the membrane and unavailable for interaction, in spite of its hydrophilic nature.

Streptococcus pyogenes

The M protein on the surface of Streptococcus pyogenes cells provides resistance of the organism to killing by phagocytic cells in the blood. Antibodies against the M protein eliminate the resistance and allow rapid removal of invadingstreptococci from the blood. Attempts to immunize humans against streptococcal infections have been impeded by the occurrence of rheumatic fever and rheumatic heart disease as toxic side effects of immunization. This was also the case when purified M protein was used for immunization. It has been conjectured that rheumatic heart disease might be caused by an immunological crossreaction between the M protein and heart tissue. Thus, it was of interest to find whether immunization could be achieved by small fragments of the M protein, thereby reducing the chance of crossreaction. Recently, Beachey et al. (33) synthesized 35- and 12-amino acid long fragments of the M protein and, by passive immunization with rabbit antibodies against these peptides, achieved effective protection for mice against streptococcal infection.

Further Examples of Antisera Against Synthetic Peptides

Antiserum against a tetradecapeptide corresponding to a region in diphtheria toxin effectively neutralized its dermonecrotic and lethal effects in guinea pigs (34). Also, a decapeptide fragment of p60src of Rous sarcoma virus, corresponding to residues 415 to 424, gave rise to antibodies that immunoprecipitated p60src and crossreacted with transforming P90 of Y 73, another chicken virus (104). Another serum against p60

src was produced using a peptide corresponding to residues 498 to 512, and these antibodies immunoprecipitated various strains of Rous sarcoma virus as well as P105, the transforming protein of chicken sarcoma virus PRCII (L.A. Rohrscheider, personal communication). An antiserum against the carboxy terminus of polio virus protein p63 was used to demonstrate that the polio virus replicase and polyuridylic acid synthetase activities are intrinsic properties of this protein (105). Branton and his collaborators successfully used synthetic peptides to produce antisera against the 58 K protein encoded by the region E1B and a series of overlapping proteins encoded by region E1A of human adenovirus type 5. The amino acid sequences for these proteins had been deduced from the nucleotide sequences of Ad5 DNA (P. Branton, personal communication). Schaffhausen et al. succeeded in raising antibodies against a nonapeptide corresponding to an internal region of polyoma virus medium T-antigen (residues 311 to 319) including the tyrosine phosphorylation site (residue 315) of this protein. Not unexpectedly, the antiserum prevented phosphorylation of tyrosine 315 by the medium T-antigen-associated kinase activity. It is interesting that the antiserum precipitates only that fraction of medium T-antigen which is unphosphorylated at tyrosine 315 (106). Finally, Harvey et al. prepared antiserum against the carboxy-terminal six amino acids of SV40 small T-antigen (107). It immunoprecipitates small T-antigen synthesized *in vivo* and *in vitro*. We raised antisera against the same hexapeptide and found that it precipitated small T-antigen more efficiently when the extracts had been pre-treated with the SH-group reagent N-ethyl maleimide (NEM), indicating that the carboxy terminus of small T-antigen may be inaccessible to the antibody and becomes exposed after NEM treatment (G. Walter and R.F. Doolittle, unpublished).

SUMMARY

After a long incubation period, the notion that synthetic peptides can be effectively used as immunogenic congeners of real proteins finally was realized by the community at large, spurred as it was by the explosion of DNA sequence data. Thus, in the last two years there has been a plethora of reports based on this technique, some identifying putative gene products, others establishing initiation and termination points, and some locating proteins in a subcellular sense. In addition to these basic research-oriented studies, there has been a barrage of studies aimed at generating synthetic peptide vaccines. How practical this latter approach may be has yet to be established, but the outlook is promising indeed.

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